AD_____

Award Number: W81XWH-08-1-0178

TITLE: Understanding and Targeting Cell Growth Networks in Breast Cancer

PRINCIPAL INVESTIGATOR: Dr. Jason Weber

CONTRACTING ORGANIZATION: Washington University
St. Louis, MO 63130

REPORT DATE: April 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

| **1. REPORT DATE** *(DD-MM-YYYY)* | **2. REPORT TYPE** | **3. DATES COVERED** *(From - To)* |
|---|---|---|
| 01-04-2011 | Annual | 17 MAR 2010 - 16 MAR 2011 |

**4. TITLE AND SUBTITLE**
Understanding and Targeting Cell Growth Networks in Breast Cancer

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-08-1-0178

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**
Dr. Jason Weber

E-Mail: JWEBER@DOM.wustl.edu.

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Washington University
St. Louis, MO 63130

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

In this third annual review, we have demonstrated that NPM and eEF1a1 5' TOP mRNAs are translationally repressed by rapamycin. The eEF1a1 5' UTR, but not the NPM 5' UTR, is sufficient to confer rapamycin sensitivity to *luciferase*. The NPM 5' TOP motif is neither necessary nor sufficient for growth-dependent translational control of the NPM mRNA. The eEF1a1 5' TOP motif functionally dominates the NPM TOP. ARF impairs association of DDX5 with the nuclear pre-ribosome fractions. Overexpression of DDX5 promotes ribosome output. ARF overexpression and DDX5 knockdown each reduce the cytosolic polysome profile in a p53-independent manner. DDX5 is a crucial non-oncogene in human breast cancer. Taken together, these insightful findings bring us significantly closer to our goal of understanding how signaling pathways impact major tumors suppressors in the control of cell growth.

**15. SUBJECT TERMS**
Cell growth, breast cancer cells, p68DDX5, ribosomes

| **16. SECURITY CLASSIFICATION OF:** | | | **17. LIMITATION OF ABSTRACT** | **18. NUMBER OF PAGES** | **19a. NAME OF RESPONSIBLE PERSON** |
|---|---|---|---|---|---|
| **a. REPORT** | **b. ABSTRACT** | **c. THIS PAGE** | | | USAMRMC |
| U | U | U | UU | 18 | **19b. TELEPHONE NUMBER** *(include area code)* |

**Table of Contents**

**INTRODUCTION**

Cancers result from an inability of a cell to control its own growth. Normally, a cell interprets external and internal signals to create a balanced growth schedule. The main interpreters of these signals within a cell are called ARF and p53, and it falls on the shoulders of these two proteins to maintain normal cell growth. In this sense, both ARF and p53 are tumor suppressors that constantly monitor the growth state of the cell. In mouse and human cancers, loss of the ARF tumor suppressor is second only to mutation of p53, providing critical evidence of ARF's role in both monitoring and preventing the outbreak of cancer cells. A common target of ARF is the NPM/B23 oncogene, an abundant protein of the nucleolus. NPM normally responds to growth factors and, due to its nucleolar localization, is thought to transmit these growth signals to the maturing ribosome machinery. Cells lacking *Arf* exhibit tremendous gains in ribosome production and subsequent protein synthesis. Moreover, the entirety of this growth phenotype is dependent on NPM and p68DDX5 expression in the nucleolus, with loss of either capable of completely reversing the phenotype back to normal. This exciting new finding indicates that ARF is a master regulator of cell growth through its tight control of NPM- or DDX5-directed ribosome production and export. Importantly, we have found NPM overexpressed in nearly 50% of breast carcinomas that we have analyzed, implying that dysregulation of NPM may be a key event in promoting breast cancer development. In effect, tumor cells that require increased protein synthesis might accumulate more NPM or DDX5 in an attempt to increase ribosome output. It is our goal to determine whether NPM directly regulates ribosome maturation to promote breast cancer formation and to establish the importance of ARF in deterring this effect. We propose to now determine the complex roles of ARF, DDX5, and NPM in the nucleolus of breast epithelial cells and how they impact both ribosome biogenesis and cell growth to prevent and/or promote tumorigenesis.

This work has tremendous clinical implications as *Arf* (9p21) and *p68Ddx5* (17q24) reside on loci that are either deleted or amplified in ER+ resistant breast tumors, respectively. This fact makes our basic science on this interesting growth network directly applicable to the breast cancer phenotype/genotype.

**BODY**

As stated in the approved Statement of Work, we focused our energies on the tasks planned for Months 25-36. These included experiments outlined in Tasks 2 and 3. In the last two months of this fiscal year (January and February 2011) we have initiated experiments in Task 4. In this third Annual Progress Report, we detail the progress and results from these studies.

*Task 2*. Examine the mechanism behind NPM's ability to promote ribosome biogenesis and cell growth in breast epithelial cells (Months 1-36):

c. Validate the responsiveness of a novel 5'-3' NPM-TOP luciferase reporter construct to in vitro mTOR signals (Months 12-36).

During the third year of this grant, we have focused our efforts more broadly on completing the experiments outlined in Tasks 2 and 3. Recognition and binding of elements within the 5' and 3' UTRs of mRNAs by regulatory proteins is a common mechanism underlying selective mRNA translational control.

Indeed, previous reports have indicated that various mRNAs are subject to such regulation. To determine whether a comparable mechanism may be responsible for the translational regulation of NPM, we first identified the 5' UTR sequence of the NPM transcript by rapid amplification of cDNA ends (RACE) (GenBank accession number GU214027). Like the human NPM 5' UTR, RACE revealed that the murine NPM 5' UTR contains a canonical terminal oligopyrimidine tract (TOP) also contained in the 5' UTRs of transcripts encoding ribosomal proteins, elongation factors, and other components of the translational machinery. We attained the complete NPM and GAPDH 3' UTR sequences from GenBank (accession numbers BC054755.1 and NM_008084.2, respectively).

We sought to evaluate whether the NPM 5' and 3' UTRs were sufficient to modulate translation of another ORF in a manner equivalent to translational regulation of the NPM ORF. Specifically, we wanted to determine whether fusion of the NPM 5' and 3' UTRs to a firefly *luciferase* (F*luc*) ORF rendered Fluc expression sensitive to rapamycin. To test this, *Tsc1*$^{-/-}$*p53*$^{-/-}$ mouse embryonic fibroblasts (MECs) were transduced with plasmids encoding NPM 5' and 3' UTR-flanked F*luc*.

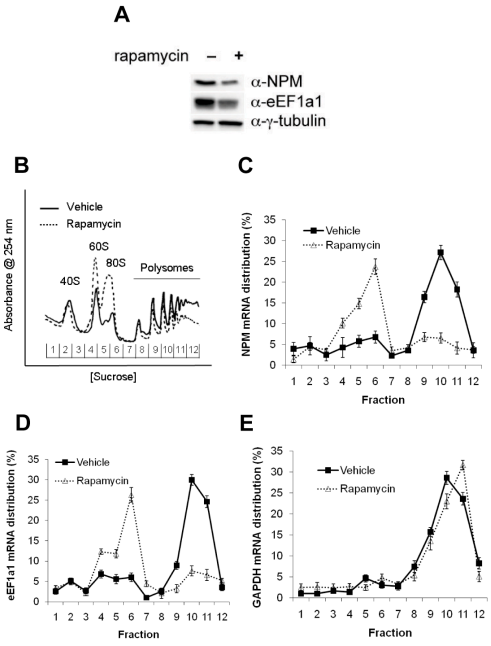



**Figure 1 NPM and eEF1a1 5' TOP mRNAs are translationally repressed by rapamycin.** *Tsc1*$^{-/-}$*p53*$^{-/-}$ MECs were treated with vehicle (–) or rapamycin (+). (**A**) NPM and eEF1a1 protein levels are decreased upon rapamycin treatment. Proteins were resolved by SDS-PAGE and analyzed by Western blot using antibodies recognizing NPM, eEF1a1, and γ-tubulin. (**B**) Polysome formation is reduced in cells treated with rapamycin. Cytosolic extracts were fractionated over a sucrose gradient, and ribosomal subunits were detected by constant UV monitoring ($A_{254nm}$) of gradients. (**C-E**) mRNA was measured by qRT-PCR from total RNA. Data are mean ± s.d. of three independent experiments. (**C**) Rapamycin results in a shift in NPM mRNA distribution from polysomes to subpolysomes. (**D**) eEF1a1 mRNAs are excluded from actively translating polysomes upon treatment with rapamycin. (**E**) Polysomal GAPDH mRNA distribution is unaltered by rapamycin.

Although sensitivity of TOP mRNA translation to rapamycin has been reported to vary from resistance to marked repression, protein levels of eEF1a1 decreased upon rapamycin treatment (Figure 1A). Consistent with previous findings from our lab, NPM protein expression also was reduced by rapamycin (Figure 1A). To further evaluate whether the eEF1a1 mRNA was comparable to NPM in response to rapamycin, we examined polysomes and the distribution of each TOP mRNA in subpolysomal and polysomal fractions. Cytosolic ribosomes were isolated by sucrose gradient

centrifugation from *Tsc1*$^{-/-}$*p53*$^{-/-}$ MECs treated with vehicle or rapamycin. As expected, rapamycin reduced polysome formation (Figure 1B). NPM mRNAs were redistributed from actively translating polysome fractions to subpolysomal fractions upon rapamycin treatment (Figure 1C). This bimodal distribution, which is characteristic of TOP mRNAs (2), was also evident for eEF1a1 mRNAs (Figure 1D). Importantly, we observed no change in the distribution of non-TOP GAPDH mRNAs upon treatment with rapamycin (Figure 1E), indicating that rapamycin-mediated inhibition of mTORC1 signalling specifically affected translation of TOP mRNAs. Taken together, these data suggested that NPM and eEF1a1 transcripts perhaps share a similar mechanism of translational control.

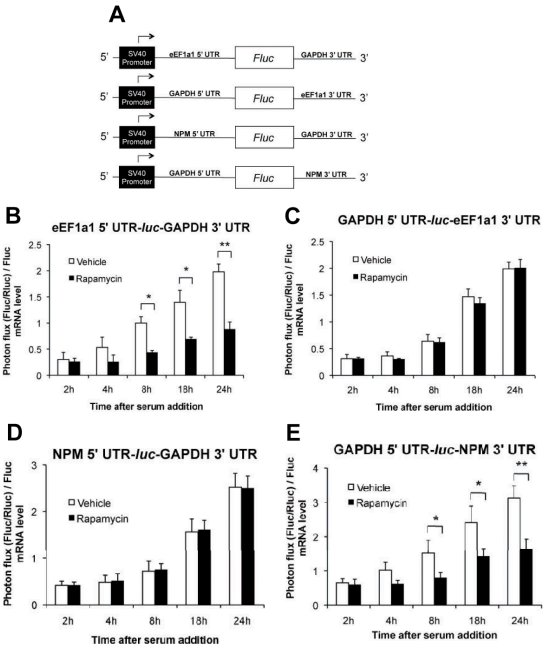
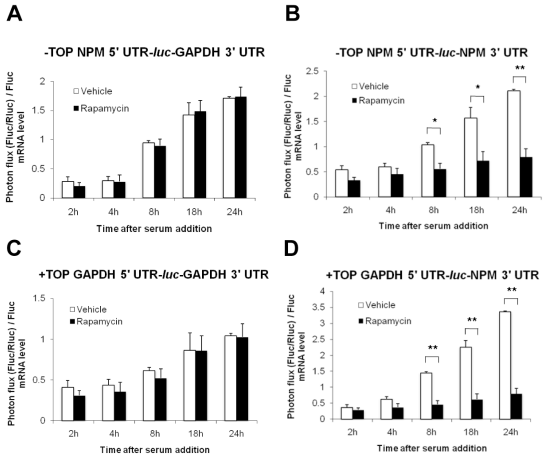



**Figure 2 The eEF1a1 5' UTR, but not the NPM 5' UTR, is sufficient to impart mTOR-mediated translational control to *luciferase*.** (**A**) Schematic representation of chimeric firefly *luciferase* reporters used to examine regulation conferred by the 5' and 3' UTRs of eEF1a1. (**B-E**) *Tsc1*$^{-/-}$*p53*$^{-/-}$ MECs were treated as described previously (1). Cells were transfected with plasmids diagrammed in A. Data shown are mean ± s.d. of three independent experiments (* $P < 0.05$, ** $P < 0.005$, Student's *t*-test). (**B**) Activity of eEF1a1 5' UTR-*luc*-GAPDH 3' UTR is attenuated by rapamycin. (**C**) GAPDH 5' UTR-*luc*-eEF1a1 3' UTR activity is unaffected upon rapamycin treatment. (**D**) Rapamycin fails to affect NPM 5' UTR-*luc*-

Given that the TOP mRNAs investigated here demonstrated rapamycin sensitivity at the level of translation, and previous reports have established the requirement of the 5' TOP motif for proper TOP mRNA translational control, we reasoned that the 5' UTRs of eEF1a1 and NPM should confer rapamycin sensitivity to a *firefly* luciferase (F*luc*) reporter. To test this, we generated chimeric reporters by fusing the eEF1a1 or NPM 5' UTR and the GAPDH 3' UTR or the GAPDH 5' UTR and the eEF1a1 or NPM 3' UTR to the respective ends of F*luc* (Figure 2A). It should be noted that the GAPDH 5' and 3' UTRs were previously found by our lab to impart no mTORC1-driven translational regulation to a reporter. To evaluate the eEF1a1 UTRs, *Tsc1*$^{-/-}$*p53*$^{-/-}$ MECs were transduced with plasmid encoding eEF1a1 5' UTR-F*luc*-GAPDH 3' UTR or GAPDH 5' UTR-F*luc*-eEF1a1 3' UTR, and Fluc protein activity was assayed. As predicted, rapamycin resulted in dramatic diminution of eEF1a1 5' UTR-Fluc-GAPDH 3' UTR protein activity (Figure 2B). Protein activity of GAPDH 5' UTR-Fluc-eEF1a1 3' UTR, however, was resistant to rapamycin (Figure 2C). Conversely, rapamycin failed to affect NPM 5' UTR-Fluc-GAPDH 3' UTR protein

activity (Figure 2D), but attenuated protein activity of GAPDH 5' UTR-Fluc-NPM 3' UTR (Figure 2E). Data that the NPM 3' UTR is sufficient to impart rapamycin sensitivity is consistent with previous work in which far upstream element (FUSE)-binding protein 1 (FBP1) was characterized by our lab as a NPM 3' UTR-binding protein that represses NPM translation upon inhibition of mTORC1. In contrast, resistance of the NPM 5' UTR to rapamycin indicates that the NPM 5' UTR, unlike the eEF1a1 5' UTR, is not sufficient to render Fluc sensitive to growth-dependent signals emanating from mTORC1. Collectively, these findings suggest that the eEF1a1 mRNA, a prototypical, representative TOP mRNA, utilizes its 5' TOP motif differently than the NPM transcript to modulate translational control.

**Figure 3 The NPM 5' TOP motif is neither necessary nor sufficient for growth-dependent translational control of the NPM mRNA.** (**A-D**) *Tsc1*$^{-/-}$*p53*$^{-/-}$ MECs were treated as previously described (1). Data shown are mean ± s.d. of three independent experiments (* $P < 0.05$, ** $P < 0.005$, Student's *t*-test). (**A-B**) The 5' TOP motif was deleted from the NPM 5' UTR (- TOP). (**A**) Activity of –TOP NPM 5' UTR-*luc*-GAPDH 3' UTR is unaffected by rapamycin. (**B**) Rapamycin attenuates –TOP NPM 5' UTR-*luc*-NPM 3' UTR activity. (**C-D**) The 5' TOP motif of the NPM mRNA was added to the GAPDH 5' UTR (+ TOP). (**C**) Rapamycin has no effect on the activity of +TOP GAPDH 5' UTR-*luc*-GAPDH 3' UTR. (**D**)




These findings led us to hypothesize that the 5' TOP motif of NPM is not essential for its translational control. To test this, we deleted the TOP motif in the NPM 5' UTR (–TOP NPM 5' UTR). We then combined the –TOP NPM 5' UTR with the NPM 3' UTR or the GAPDH 3' UTR by fusing the UTRs to the respective ends of F*luc*. *Tsc1*$^{-/-}$*p53*$^{-/-}$ MECs were transduced with plasmid encoding –TOP NPM 5' UTR-F*luc*-GAPDH 3' UTR. Protein activity of –TOP NPM 5' UTR-F*luc*-GAPDH 3' UTR increased as a function of serum stimulation but was unaffected by rapamycin (Figure 3A). In contrast, –TOP NPM 5' UTR-F*luc*-NPM 3' UTR protein induction was significantly attenuated in the presence of rapamycin versus vehicle (Figure 3B). Notably, these data are comparable to findings from cells transduced with plasmid encoding TOP-containing NPM 5'UTR-F*luc*-GAPDH 3' UTR (Figure 2D) and TOP-containing NPM 5'UTR-F*luc*-NPM 3'

UTR and subjected to the same assay. Together, these data indicate that the 5' TOP motif is not necessary for translational regulation of the NPM mRNA.

Given our findings that the 5' TOP is not required for translational control of the NPM transcript, we questioned whether the TOP motif of NPM is sufficient to confer regulatory properties to another mRNA. To test this, we added the NPM 5' TOP motif to the 5' end of the GAPDH 5' UTR (+TOP GAPDH 5' UTR). As described above, we then fused the +TOP GAPDH 5' UTR to the 5' end of F*luc*. The GAPDH 3' UTR or the NPM 3' UTR were fused to F*luc* at the 3' end, and *Tsc1*$^{-/-}$*p53*$^{-/-}$ MECs were transduced with plasmid encoding +TOP GAPDH 5' UTR-F*luc*-GAPDH 3' UTR or +TOP GAPDH 5' UTR-F*luc*-NPM 3' UTR. Protein activity of +TOP GAPDH 5' UTR-Fluc-GAPDH 3' UTR was resistant to rapamycin-induced repression (Figure 3C). Rapamycin, however, dramatically diminished +TOP GAPDH 5' UTR-Fluc-NPM 3' UTR protein activity (Figure 3D). Collectively, these data demonstrate that the TOP motif is neither necessary for translational regulation of the NPM mRNA nor is it sufficient to confer rapamycin sensitivity to the GAPDH 5' UTR. Instead, it appears that modulation of NPM translation depends exclusively on regulatory elements within the NPM 3' UTR.

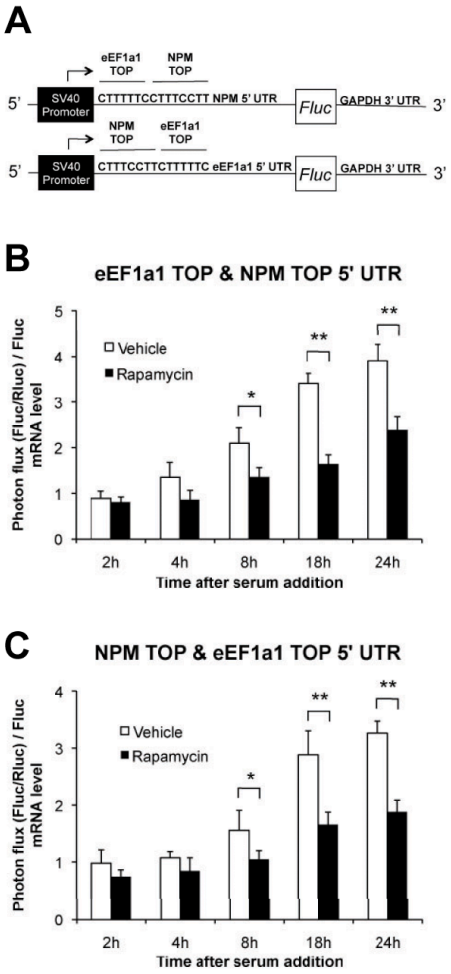



**Figure 4 The eEF1a1 5' TOP motif functionally dominates the NPM TOP.** (**A**) Schematized depictions of firefly *luciferase* reporters used to evaluate 5' UTRs containing NPM and eEF1a1 TOP motifs in combination. (**B-C**) *Tsc1*$^{-/-}$*p53*$^{-/-}$ MECs were treated as described (1) and were transduced with plasmids depicted in A. Data shown are mean ± s.d. of three independent experiments (* $P < 0.05$, ** $P \leq 0.005$, Student's *t*-test). (**B**) Rapamycin reduces eEF1a1 TOP & NPM TOP 5' UTR-*luc*-GAPDH 3' UTR activity. (**C**) Activity of NPM TOP & eEF1a1 TOP 5' UTR-*luc*-GAPDH 3' UTR is diminished by rapamycin treatment.

Our surprising findings that the 5' TOP motif of NPM is neither necessary nor sufficient for mTORC1-mediated translational regulation of the NPM mRNA led us to question whether the NPM TOP inhibits rapamycin sensitivity or is instead functionally inert. To investigate this, we added the 5' TOP of eEF1a1 to the wild-type (WT) NPM 5' UTR, thereby placing the eEF1a1 TOP immediately upstream of the NPM TOP (Figure 4A; designated as eEF1a1 TOP & NPM TOP 5' UTR). Conversely, we appended the NPM TOP motif to the WT eEF1a1 5' UTR, which put the eEF1a1 TOP proximally downstream of the NPM TOP (Figure 4A; labelled NPM TOP & eEF1a1 TOP 5' UTR). These 5' UTR mutants were fused to the 5' end of F*luc*, and the GAPDH 3' UTR was fused to the F*luc* 3' end (Figure 4A).

If the 5' TOP motif of NPM promotes rapamycin resistance, then its addition to the WT eEF1a1 5' UTR should attenuate rapamycin sensitivity. Alternatively, if the NPM TOP is functionally inactive, then rapamycin sensitivity conferred by the WT eEF1a1 5' UTR (Figure 2B) should be maintained, and addition of the eEF1a1 TOP to the WT NPM 5' UTR should impart mTORC1-dependent translational control rather than be nullified by the NPM TOP. We transduced *Tsc1*$^{-/-}$*p53*$^{-/-}$ MECs with plasmid

encoding eEF1a1 TOP & NPM TOP 5' UTR-F*luc*-GAPDH 3' UTR or NPM TOP & eEF1a1 TOP 5' UTR-F*luc*-GAPDH 3' UTR and assayed Fluc protein activity. Addition of the eEF1a1 TOP to the WT NPM 5' UTR resulted in rapamycin-induced diminishment of eEF1a1 TOP & NPM TOP 5' UTR-F*luc*-GAPDH 3' UTR protein activity (Figure 4B), indicating that the eEF1a1 TOP is sufficient to render the NPM 5' UTR rapamycin-sensitive. Moreover, protein activity of NPM TOP & eEF1a1 TOP 5' UTR-F*luc*-GAPDH 3' UTR exhibited rapamycin sensitivity (Figure 4C). Taken together, these data demonstrate that the NPM TOP is biologically inert, allowing for functional dominance by the eEF1a1 5' TOP motif.

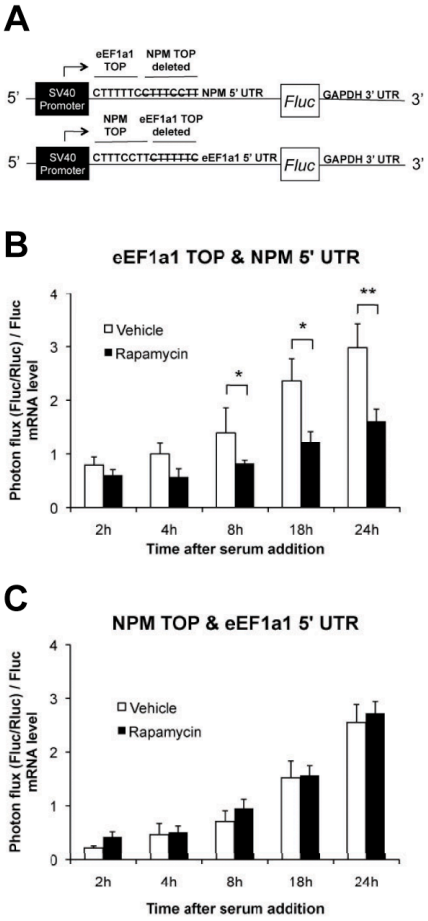



**Figure 5 Rapamycin sensitivity is dependent on the eEF1a1 TOP and independent of downstream eEF1a1 or NPM 5' UTR sequences.** (**A**) Diagrams of firefly *luciferase* reporters generated to evaluate NPM and eEF1a1 5' UTRs comprising endogenous TOP deletions. (**B-C**) *Tsc1*$^{-/-}$*p53*$^{-/-}$ MECs were treated as described (1) and were transfected with plasmids shown in A. Data shown are mean ± s.d. of three independent experiments (* $P \leq 0.05$, ** $P < 0.005$, Student's *t*-test). (**B**) Activity of eEF1a1 TOP & NPM 5' UTR-*luc*-GAPDH 3' UTR is reduced by rapamycin. (**C**) NPM TOP & eEF1a1 5' UTR-*luc*-GAPDH 3' UTR activity is

Simultaneous presence of both the eEF1a1 and NPM 5' TOPs in the experiments above provided a model for evaluation of relative 5' TOP motif function, but they precluded independent TOP examination and excluded the putative regulatory contribution of non-TOP 5' UTR sequence elements. To explore this, we deleted the endogenous TOPs from the NPM and eEF1a1 5' UTRs and added the eEF1a1 5' TOP or the NPM 5' TOP, respectively (Figure 5A; designated eEF1a1 TOP & NPM 5' UTR or NPM TOP & eEF1a1 5' UTR). As in above experiments, we fused these NPM and eEF1a1 5' UTR TOP-swapped mutants to the F*luc* 5' end, and the GAPDH 3' UTR was fused to the 3' end (Figure 5A). *Tsc1*$^{-/-}$ *p53*$^{-/-}$ MECs were transduced with plasmid encoding eEF1a1 TOP & NPM 5' UTR-F*luc*-GAPDH 3' UTR or

NPM TOP & eEF1a1 5' UTR-F*luc*-GAPDH 3' UTR, and Fluc protein activity was evaluated. In the presence of rapamycin, eEF1a1 TOP & NPM 5' UTR-F*luc*-GAPDH 3' UTR protein activity was diminished relative to vehicle treatment (Figure 5B). However, protein activity of NPM TOP & eEF1a1 5' UTR-F*luc*-GAPDH 3' UTR displayed resistance to rapamycin (Figure 5C). These findings indicate that the eEF1a1 5' TOP is necessary and sufficient to render F*luc* translationally sensitive to inhibition of mTORC1. Furthermore, these data demonstrate that 5' UTR sequences downstream of the 5' TOP motifs do not significantly affect growth-dependent regulation of either NPM or eEF1a1 mRNA translation.

*Task 3.* Establish the oncogenic potential of the p68DDX5 RNA helicase (Months 24-48).

a. Determine whether p68 is required for ribosome biogenesis (Months 24-36).

The nucleolar localization of DDX5, along with its function as an RNA helicase, suggested that DDX5 might be involved in the biogenesis of rRNA. The regulation of DDX5 localization by basal ARF led us to investigate whether ARF could control ribosome biogenesis through regulation of DDX5 function. Both p19ARF (mouse) and p14ARF (human) negatively regulate transcription of rRNA (3-5), and DDX5 has been ascribed roles as a transcriptional regulator (6). However, it is unknown whether DDX5 regulates transcription at nucleolar rDNA loci. We conducted chromatin immunoprecipitation experiments to determine whether DDX5 associated with the rDNA promoter at two previously identified binding sites of the RNA polymerase I transcription factor UBF (7). ARF regulated DDX5 association with these sites, such that DDX5 occupancy at the rDNA promoter was over two-fold greater in *Arf*-/- MECs compared to wild-type MECs (Fig. 6a). Additionally, DDX5 has been shown to be involved in processing of the 5.8S rRNA (8) and the 28S rRNA from their respective rRNA precursors (9). By immunoprecipitation, we observed a specific interaction between DDX5 and the 28S and 18S rRNA species in the lysates of *Arf*-/- MECs (Fig. 6b). This association with mature rRNA suggests that DDX5 could be involved at multiple stages in the production and assembly of ribosomes. Interestingly, in wild-type MECs the interaction of rRNA with DDX5 was decreased, suggesting that ARF can inhibit this association as well. We hypothesized that ARF may interfere with the ability of DDX5 to stimulate ribosome biogenesis by impeding access of DDX5 to maturing pre-ribosomes. Nuclear lysates obtained from equal numbers of wild-type and *Arf*-/- MECs were separated by sucrose gradient centrifugation. Enhanced association of DDX5 with the 40S and 60S pre-ribosomal fractions was observed in the *Arf*-/- nuclear lysates relative to the corresponding wild-type fractions (Fig. 6d).

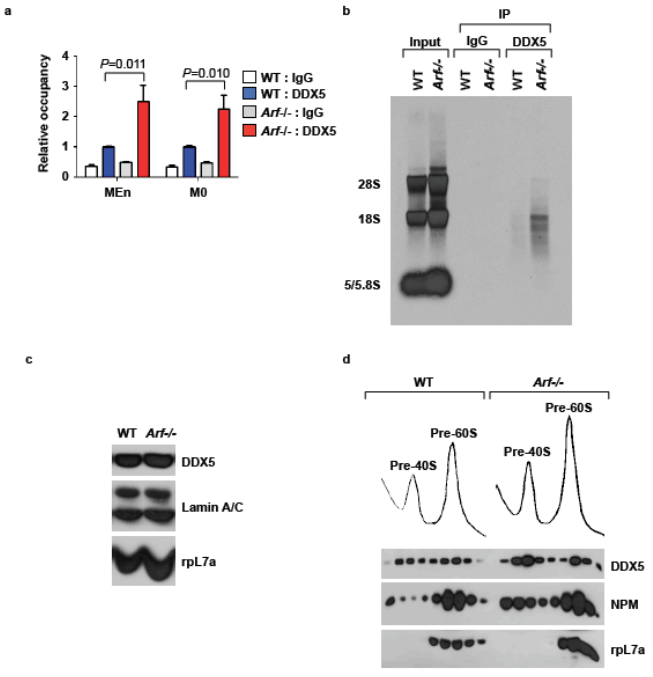



**Figure 6. ARF impairs association of DDX5 with the nuclear pre-ribosome fractions**. **(a)** Wild-type and *Arf*-/- MECs were cross-linked in paraformaldehyde and whole cell lysates were collected for chromatin immunoprecipitation using a DDX5 antibody or a normal rabbit IgG control. Quantitative PCR with primers flanking two regions, MEn and M0, on the rDNA promoter was used to amplify DNA isolated from the immunoprecipitates. **(b)** Wild-type and *Arf*-/- MECs were labeled with [methyl-$^{3}$H]-methionine for 4 hours, lysed in NET2 buffer, and DDX5 was immunoprecipitated. RNA was isolated from the DDX5 immunoprecipitate, separated on an agarose gel, and transferred to a nylon membrane. Radiolabeled RNA was visualized by autoradiography. **(c)** Equal volumes of nuclear extract from wild-type and *Arf*-/- MECs were analyzed by western blot using antibodies recognizing DDX5, Lamin A/C and rpL7a. **(d)** Equal numbers of wild-type and *Arf*-/- MECs were fractionated and nuclear extracts were subjected to sucrose density centrifugation. RNA absorbance was monitored at 254 nm as samples were fractionated and collected. Protein was precipitated and isolated from nuclear extract fractions and samples were analyzed by western blot using antibodies recognizing DDX5, NPM and rpL7a.

In order to determine whether DDX5 could accelerate ribosome biogenesis, wild-type MECs were transduced with a Flag-epitope-tagged DDX5 or a mutant (K144N) deficient in ATP binding (Fig. 7a). The K144N mutation in the Walker A motif abrogates not only ATP binding, but also the ATPase and helicase activities of DDX5 (9). The earliest observed effect of DDX5 on ribosome biogenesis was at the level of 47S pre-rRNA transcription, where both Flag-DDX5 and Flag-DDX5-K144N increased the amount of 47S

transcript per cell (Fig. 7b). The ability of DDX5 to regulate transcription of the 47S pre-RNA concurred with its aforementioned association at the rDNA promoter. Monitoring the processing of the 47S pre-rRNA transcript by pulse-chase analysis, we discovered a more rapid accumulation of mature 28S and 18S rRNAs in cells expressing Flag-DDX5 or Flag-K144N versus vector-transduced cells (Figs. 7c and d). To determine whether the accelerated production of rRNA equated with increased protein synthesis, cytosolic fractions were collected for ribosome profile analysis. Both Flag-DDX5 and Flag-DDX5-K144N enhanced the amplitude of the actively translating polyribosome fraction (Fig. 7e), indicating that ectopic expression of Flag-DDX5 ultimately increases ribosome availability for translation, and that helicase activity is not required for this induction. These results indicate that DDX5 stimulates the production of functional ribosomes by increasing the total amount of mature rRNA.

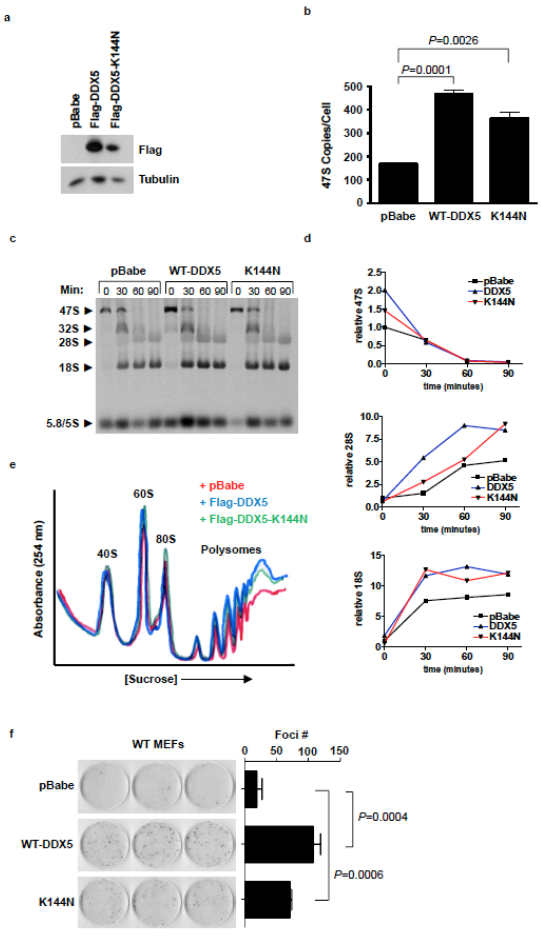



**Figure 7**. **Overexpression of DDX5 promotes ribosome output.** Wild-type MECs were transduced with empty vector or Flag-DDX5 retroviruses. **(a)** Flag immunoblot demonstrates expression of the retroviral fusion protein in whole cell lysate. **(b)** Total RNA was extracted from transduced cells and analyzed by quantitative PCR to determine copy number of the 47S pre-rRNA transcript. **(c)** Cells were labeled with [methyl-$^{3}$H]-methionine and chased for the indicated times. Radiolabeled RNA was visualized by autoradiography. **(d)** Band intensities were determined for the rRNA species in the processing assay. 47S, 28S, and 18S rRNAs were individually normalized to the pBabe sample at t=0 and tracked throughout the time course. **(e)** Wild-type MECs were infected with Flag-DDX5, Flag-DDX5-K144N, or empty vector. Equal numbers (2.5 x $10^{6}$) of cells were collected and cytosolic extracts were loaded onto sucrose density gradients and separated by centrifugation. Ribosome profiles were obtained by continuously measuring the absorbance of RNA at 254 nm. **(f)** Infected wild-type MEFs were plated at $10^{3}$ cells per dish and grown for 12 days. Foci were fixed in methanol and stained with Giemsa.

Further, the enhanced ribosome biogenesis caused by DDX5 overexpression corresponds to an increased proliferative capacity as evidenced by the ability of Flag-DDX5 and Flag-DDX5-K144N to stimulate foci formation in wild-type MEFs (Fig. 7f).

The nucleolar activities of DDX5 and p19ARF oppose one another. DDX5 stimulates ribosome production, whereas ARF inhibits ribosome biogenesis at several stages: 47S transcription, rRNA processing, and rRNA export (3, 5, 10). Ultimately, the effects of *Arf* loss are exhibited by the enhanced ribosome profiles of *Arf*-/-MECs relative to wild-type MECs (3). It was unclear, however, whether these effects of ARF on the cellular ribosome profile were truly p53-independent. To characterize the p53-independent functions of ARF on ribosome biogenesis, we utilized TKO (*p53*-/-; *Mdm2*-/-; *Arf* -/-) MECs, in which the entire ARF-Mdm2-p53 axis has been removed (11). By adding ARF back into TKO MECs we investigated growth-inhibitory effects of ARF that are completely independent of p53. HA-ARF expression reduced cytosolic ribosomes, most notably in the actively translating polyribosome fraction (Fig. 8a), demonstrating a p53-independent role for ARF in the regulation of ribosome output. Knockdown of DDX5 in TKO MECs mimicked the effects of ARF overexpression on cytosolic ribosome content (Fig. 8b), causing a notable decrease in polyribosome peak amplitude. Thus, a DDX5 loss-of-function is equivalent to a p53-independent ARF gain-of-function on ribosome output.

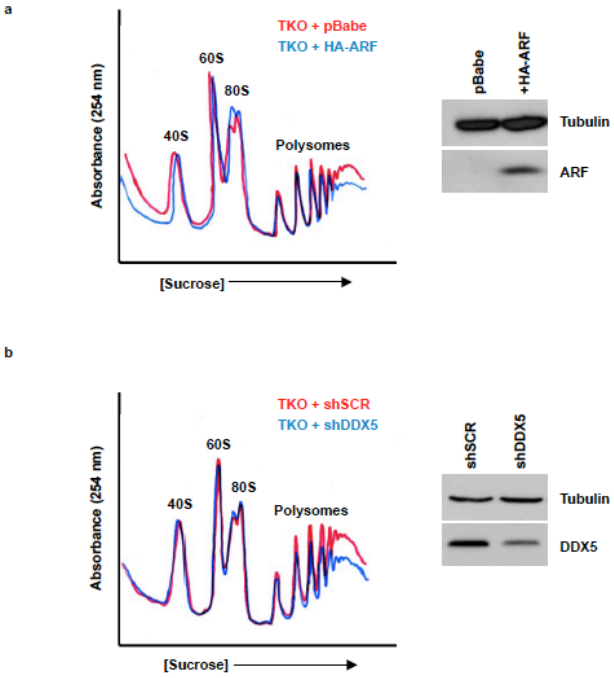
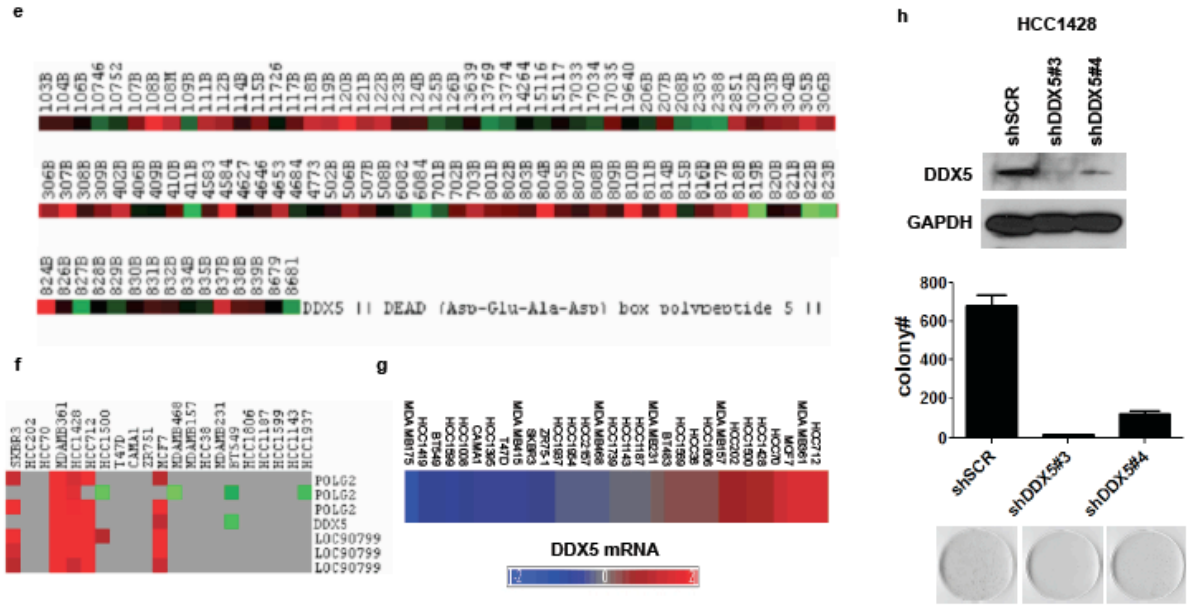



**Figure 8. ARF overexpression and DDX5 knockdown each reduce the cytosolic polysome profile in a p53-independent manner.** TKO (*Arf*/*p53*/*Mdm2*-/-) MECs infected with viruses encoding **(a)** HA-ARF or **(b)** shDDX5 were fractionated and cytosolic extracts were loaded onto a sucrose density gradient. Following high speed centrifugation, samples were monitored for the absorbance of RNA at 254 nm. Absorbance readings between samples were overlaid to compare RNA peaks corresponding to ribosomal RNA. (Side panels) Expression of ARF and DDX5 were assessed by western blot.

b. Examine the role of p68 in breast cancer cell growth (Months 36-48).

Using array CGH, we observed frequent amplification of the *DDX5* locus in relapsed estrogen receptor-positive (ER+) human breast tumors (Fig 9e) and ER+ breast cancer cell lines (Fig 9f). Microarray analysis confirmed that DDX5 mRNA expression was elevated in cells with gene amplification (Figure 9g). HCC1428 cells, which exhibit *DDX5* amplification, were transduced with siRNAs targeting DDX5 to evaluate whether the growth-inhibitory effects of DDX5 loss in MECs could be extended to human breast cancer cells. Similar to observations in MECs, reduction of DDX5 in estrogen-stimulated HCC1428 cells inhibited foci formation in a dose-dependent manner (Figure 9h). This suggests that amplification of DDX5 in human breast cancers may drive growth and proliferation and that DDX5 may be a viable non-oncogene target in a subset of ER+ tumors.

**Figure 9. Importance of DDX5 in human breast cancer**. Array CGH determination of *DDX5* gene copy number was determined over a panel of relapsed ER+ human breast tumors **(e)** and breast cancer cell lines **(f)**. **(g)** Microarray analysis of DDX5 mRNA expression was performed on a panel of human breast cancer cell lines. Data were clustered by expression level and set relative to a pooled universal reference. **(h)** HCC1428 cells were transduced to express shRNAs against DDX5 or a scrambled sequence. $10^4$ cells were plated in 10 $cm^2$ dishes and grown for 24 days in the presence of 10 nM estradiol to assess foci formation.



**KEY RESEARCH ACCOMPLISHMENTS**

- NPM and eEF1a1 5' TOP mRNAs are translationally repressed by rapamycin (Task 2c)
- The eEF1a1 5' UTR, but not the NPM 5' UTR, is sufficient to confer rapamycin sensitivity to *luciferase* (Task 2c)
- The NPM 5' TOP motif is neither necessary nor sufficient for growth-dependent translational control of the NPM mRNA (Task 2c)
- The eEF1a1 5' TOP motif functionally dominates the NPM TOP (Task 2c)
- ARF impairs association of DDX5 with the nuclear pre-ribosome fractions (Task 3a)
- Overexpression of DDX5 promotes ribosome output (Task 3a)
- ARF overexpression and DDX5 knockdown each reduce the cytosolic polysome profile in a p53-independent manner (Task 3a)
- DDX5 is a crucial non-oncogene in human breast cancer (Task 3c)

**REPORTABLE OUTCOMES**

Manuscripts:

Olanich ME, Moss BL, Piwnica-Worms D, Townsend RR, & **Weber JD** (2011). Identification of FUSE binding protein 1 as a regulatory mRNA-binding protein that represses nucleophosmin translation. *Oncogene* 30(1):77-86.

Dong Y, Li A, Wang J, **Weber JD,** and Michel LS (2010). Synthetic lethality through combined notch-epidermal growth factor receptor pathway inhibition in basal-like breast cancer. *Cancer Research*, 70: 5465-74.

Abstracts/Presentations: None

Patents/Licenses: None

Animal Models: In the second year, we have generated *Npm1*+/-*Arf*-/- and *Ddx5*+/-*Arf*-/- mice, which will be free to any research that requests them.

Cell Lines: We have developed a unique primary mouse mammary epithelial cell (MMEC) line lacking the ARF tumor suppressor. These were established directly from *Arf* knockout mice on a pure C57Bl6 background. The *Arf*-null MMECs maintain a diploid phenotype and wild-type p53. These cells are spontaneously immortal and contain no artificial genes or plasmid constructs.

Funding Applied for: None

Employment Opportunities: None.

**CONCLUSION**

We have successfully modeled NPM translation using a luciferase-based readout assay. By flanking the luciferase open reading frame with both the 5' and 3' UTRs of murine NPM, we have generated a transcript that is controlled at the level of translation by NPM sequences. We now understand the regulation of NPM translation in the presence and absence of hyperactive mTOR signals. We have shown a clear mechanism of NPM translation by the 3'-UTR binding by the FBP1 repressor and have now shown that the 5-TOP sequence of NPM is not a canonical TOP motif and is not sensitive to mTOR signaling. While the precise mechanism of FBP1 translational repression of NPM remains to be determined, we are confident that our unique reporter construct will provide critical insights for our planned experiments in *Nf1*-/- astrocytes and certainly other systems.

Our results provide a new perspective for understanding the tumor suppressor function of ARF, which has classically been thought of as a checkpoint sensor of hyperproliferative signals. The data presented here suggest that an equally important mechanism by which ARF functions as a tumor suppressor is to limit ribosome output as a defense against oncogene activation and the attendant enhanced cellular protein requirements. Whereas loss of *Arf* results in a cellular environment permissive toward oncogenic transformation, knockdown of DDX5 can reduce susceptibility to transformation. Therefore, in the absence of *Arf*, DDX5 becomes a requisite non-oncogene effector that promotes an increased translational output in accord with the higher demand for protein production required upon oncogene activation. The ability of ectopic DDX5 expression to stimulate ribosome biogenesis and growth in wild-type MECs further proves the central role of DDX5 in regulating this translational output. The inability of basal ARF to suppress the effects of DDX5 overexpression suggests an antagonistic relationship, where DDX5 loss-of-function phenocopies an ARF gain-of-function and *vice versa*.

Our data showing the growth-stimulatory functions of DDX5 in ribosome biogenesis provides a strong rationale to explain the link between DDX5 and cancer. Although still in its infancy, most non-oncogenes are thought of as critical regulators of cellular stress responses and that their expression provides cancer cells the means to tolerate multiple stresses (12). It is unclear how DDX5 and ribosome biogenesis fits into this stress tolerance model. Rather, DDX5 may represent a class of non-oncogenes whose activities are unleashed in the absence of crucial tumor suppressors. In this setting, the role of the DDX5 non-oncogene is to make a required cellular process, such as ribosome biogenesis, more efficient or prolific in preparation for the tremendous protein synthesis demands following malignant transformation. It remains to be determined whether DDX5 will be an efficacious target in the treatment of cancer; however our results validate its importance in supplying the sustained ribosome output required for oncogenic transformation. In summary, DDX5 participation in ribosome biogenesis is negatively regulated by ARF, which inhibits the DDX5-NPM interaction, suggesting a dynamic interplay through which ARF and DDX5 duel for nucleolar growth control.

**REFERENCES**


1. Olanich ME, Moss BL, Piwnica-Worms D, Townsend RR, & Weber JD (2011) Identification of FUSE-binding protein 1 as a regulatory mRNA-binding protein that represses nucleophosmin translation. *Oncogene* 30(1):77-86.
2. Meyuhas O (2000) Synthesis of the translational apparatus is regulated at the translational level. *Eur J Biochem* 267(21):6321-6330.
3. Apicelli AJ*, et al.* (2008) A non-tumor suppressor role for basal p19ARF in maintaining nucleolar structure and function. *Mol Cell Biol* 28(3):1068-1080.
4. Ayrault O*, et al.* (2006) Human tumor suppressor p14ARF negatively regulates rRNA transcription and inhibits UBF1 transcription factor phosphorylation. *Oncogene* 25(58):7577-7586.
5. Lessard F*, et al.* (2010) The ARF tumor suppressor controls ribosome biogenesis by regulating the RNA polymerase I transcription factor TTF-I. *Mol Cell* 38(4):539-550.
6. Fuller-Pace FV (2006) DExD/H box RNA helicases: multifunctional proteins with important roles in transcriptional regulation. *Nucleic Acids Res* 34(15):4206-4215.
7. O'Sullivan AC, Sullivan GJ, & McStay B (2002) UBF binding in vivo is not restricted to regulatory sequences within the vertebrate ribosomal DNA repeat. *Mol Cell Biol* 22(2):657-668.
8. Fukuda T*, et al.* (2007) DEAD-box RNA helicase subunits of the Drosha complex are required for processing of rRNA and a subset of microRNAs. *Nat Cell Biol* 9(5):604-611.
9. Jalal C, Uhlmann-Schiffler H, & Stahl H (2007) Redundant role of DEAD box proteins p68 (Ddx5) and p72/p82 (Ddx17) in ribosome biogenesis and cell proliferation. *Nucleic Acids Res* 35(11):3590-3601.
10. Sugimoto M, Kuo ML, Roussel MF, & Sherr CJ (2003) Nucleolar Arf tumor suppressor inhibits ribosomal RNA processing. *Mol Cell* 11(2):415-424.
11. Weber JD*, et al.* (2000) p53-independent functions of the p19(ARF) tumor suppressor. *Genes Dev* 14(18):2358-2365.
12. Luo J, Solimini NL, & Elledge SJ (2009) Principles of cancer therapy: oncogene and non-oncogene addiction. *Cell* 136(5):823-837.


**APPENDICES**

None